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Effects of melatonin in isolated rat papillary muscle

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Abstract **Melatonin (*N*-acetyl-5-methoxytryptamine), the principal hormone of the vertebral pineal gland, elicits several neurobiological effects. However, the effects of melatonin on cardiac muscle are still unknown. The first goal of the study was to investigate the role of melatonin on myocardial contractility in isolated rat papillary muscle using dose-response curves to melatonin, to isoproterenol and calcium either in the presence or in the absence of melatonin (0.3 nM). Response curves to isoproterenol were additionally performed in the presence of melatonin plus the specific receptor antagonist *N*-acetyltryptamine (10 μM); the adenylate-cyclase stimulator forskolin (10 μM) was also used.**

Melatonin has no direct inotropic effect in isolated rat papillary muscle but counteracts isoproterenol but not [Ca^{2+}] effects. In fact, the EC_{50} for isoproterenol was significantly higher in the presence than in the absence of melatonin ($p < 0.001$). This anti-adrenergic action occurs through an interaction to a specific cardiac receptor. Forskolin-stimulated adenylate cyclase induced an increase of contractile force (+118 ± 25%) which was reduced in the presence of melatonin (+26 ± 10%; $p < 0.01$).

In conclusion, we found that melatonin possess anti-adrenergic effect in isolated rat papillary muscle. This phenomenon was abolished in the presence of its receptor antagonist *N*-acetyltryptamine demonstrating that melatonin operates through a specific cardiac receptor. The reduction of contractility increase, induced by forskolin-stimulated adenylate cyclase, shows that melatonin may act through a reduction of cyclic AMP accumulation.

Key words: Melatonin; Adreno-receptors; Myocardial contractility; Isoproterenol; Calcium; *N*-Acetyltryptamine; Forskolin; Papillary muscle

1. Introduction

Increased interest has recently gained melatonin (*N*-acetyl-5-methoxytryptamine) since it was suggested that it is involved in aging processes [1–5]. Melatonin is a circulating hormone that serves as an endogenous timer for mammalian circadian rhythms, and it may regulate the reproductive alterations occurring in response to changes in day length seasonally [6,7].

Melatonin exerts its action through pharmacologically specific guanine nucleotide binding protein coupled-receptors designated as Mel_{1a} and Mel_{1b} in various tissues including retina, brain, liver, spleen, and pituitary gland [8,9]. Recent studies of Iuvone and Gan indicate that melatonin receptors are coupled to inhibitory G proteins to cyclic AMP accumulation [10] and to dopamine receptor-regulated adenylate cyclase [11].

The exact action of melatonin in cardiac muscle is still unknown. Saturable and reversible 2-$^{[125]}$ iodomelatonin binding sites has been demonstrated in heart membrane preparation [12]. Intravenous melatonin administration in rats produced a dose related-fall in mean arterial pressure and heart rate abolished by pretreatment with bilateral vagotomy [13]. This latter study suggests that melatonin inhibits sympathetic nervous system. The hypothesis of an anti-adrenergic effect of melatonin is also supported in other experimental studies [14,15].

The goal of the present study was to investigate the effect of melatonin in isolated rat papillary muscle. We here report that melatonin has no direct inotropic effect on cardiac contractility. More importantly is that the dose-response curve to isoproterenol was shifted to the right in the presence of melatonin, and this phenomenon was mediated by a specific receptor-interaction on the heart. Our study supports the concept that melatonin has an anti-adrenergic action in the heart.

2. Methods

2.1. Isolated papillary muscle

Male normotensive Wistar-Kyoto rats weighting 290 ± 30 gr were studied ($n = 22$). The rats were killed by cervical dislocation, the hearts were quickly excised and anterior papillary muscles dissected from left ventricle and transferred to a tissue bath. The dissected papillary muscle ranged from 0.5 to 0.9 mm^2 as cross-sectional area and 2–3 mm in length, as previously described in details [16]. Muscles with cross-sectional area < 0.5 or > 1.5 mm^2 were excluded from analysis. The papillary muscles were superfused at 30°C with oxygenated (95% CO_2, 5% O_2) Tyrode solution with the following composition (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, $CaCl_2$ 1 and glucose 11.5. The papillary muscle was tied by means of a short silk thread to a stainless steel rod attached to a force transducer (Harvard Apparatus; mod. 52-9545, 50–60 Hz). The other end of the strand was immobilized by means of one of the stainless steel pins used as stimulating electrodes. The muscle portion between the distal end of the muscle, connected to a force transducer and delimited by L-shaped pin, could move freely during contraction. The papillary muscle was continuously driven at a frequency of 60 beats/min (1 Hz) by means of electrical stimuli (5 msec pulses at a voltage 10% above threshold) delivered by a WPI stimulator (Mod. A 310 Accapulser). The stimulated papillary muscles were stretched to approximately 30% of their resting length and were equilibrated for a minimum of 1 hr in Tyrode solution prior to the experiments. Tension in response to excitation was normalized for muscle cross-sectional area in square millimetres (g/mm^2) [16]. Resting tension (determined by initial muscle length), active tension (i.e. the tension developed during isometric contraction), and developed tension (active tension minus resting tension) were evaluated.

2.2. Response to melatonin, isoproterenol and [Ca^{2+}]

Concentration response curves to melatonin were constructed in an incremental fashion (from 0.01 to 100 nM of final concentration).

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Concentration response curves to isoproterenol and [Ca^{2+}] were constructed in an incremental fashion in presence or absence of melatonin (0.3 nM). We used the 0.3 nM concentration of melatonin on the basis of preliminary experiments which showed similar effects in a range comprised between 0.1 and 1 nM. This concentration was selected also because it represents the physiological nocturnal peak observed in humans [17]. A maximum response both to isoproterenol and [Ca^{2+}] was judged when there was no increase in contraction amplitude with increasing concentrations, or when toxic signs such a phasic contractions or decreases in diastolic length occurred. Concentration-response curves were used only if changes were fully reversible on washout. The EC_{50} value (concentration for half maximal effect) for isoproterenol and [Ca^{2+}] in presence or absence of melatonin were calculated for each papillary muscle.

2.3. Response to N-acetyltryptamine and forskolin

Concentration response curve to isoproterenol were constructed in an incremental fashion in the presence or absence of melatonin (0.3 nM) plus the specific receptor antagonist *N*-acetyltryptamine (10 μM). This dose was selected on the basis of our preliminary experiments and by previous experience in other experimental models [10]. The EC_{50} value (concentration for half maximal effect) for isoproterenol in the presence or absence of melatonin (0.3 nM) plus the specific receptor antagonist was calculated for each papillary muscle. Since it is well known that isoproterenol induces cyclic AMP increase in the rat papillary muscle model [18,19], we performed experiments in the presence of the adenylate-cyclase stimulator forskolin (10 μM) [10] in the presence and absence of 0.3 nM melatonin.

2.4. Materials

Melatonin (#cod. M5250), (−)-isoproterenol, and forskolin were obtained from Sigma Chemicals (Milan, Italy). Melatonin was solubilized in ethanol (0.01%). *N*-Acetyltryptamine was obtained from ICN Pharmaceuticals (Costa Mesa, CA; #cod. 159685). Controls were carried out in the presence of the same amount of ethanol (0.01%) used to solve melatonin.

2.5. Data recording and statistical analysis

Contractile force were recorded simultaneously and continuously at slow speed (0.05 mm/sec) with a Gould recorder (Model 2400), as previously described [16]. The results are reported as mean values ± standard deviation and were analyzed with paired *t*-test. Isoproterenol curves were analyzed by the iterative non-linear least-square regression program (NFIT). The percent modification of developed tension in dose-response curve to melatonin was performed by considering 0% the contractile force at baseline while the end-scale arbitrary goes up to 100%. Values less than 0.05 ($p < 0.05$) were considered significant.

3. Results

Our results primarily demonstrate that melatonin does not exert direct inotropic effect in isolated rat papillary muscle. Concentration response curve to melatonin shows that developed tension did not change in controls compared to muscle exposed to melatonin (Fig. 1, $n = 7$). Furthermore, Table 1

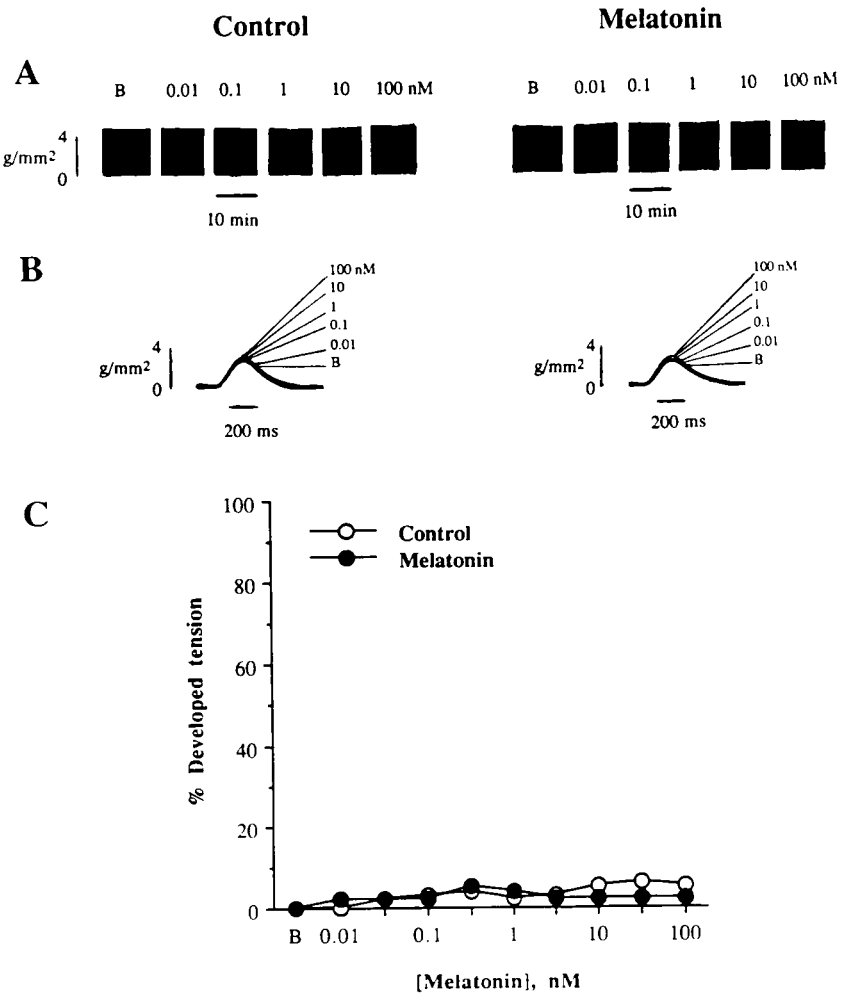

Fig. 1. Dose-response effects of melatonin (diluted in ethanol 0.01%, right) and control (ethanol 0.01%, left) on twitch tension of rat papillary muscle (A). (B) Superimposed twitches obtained from the experiments illustrated in A. This figure is representative of data obtained in seven other experiments. (C) Dose-response curve to control and melatonin on percent developed tension. Results are the mean ± S.E. ($n = 7$).

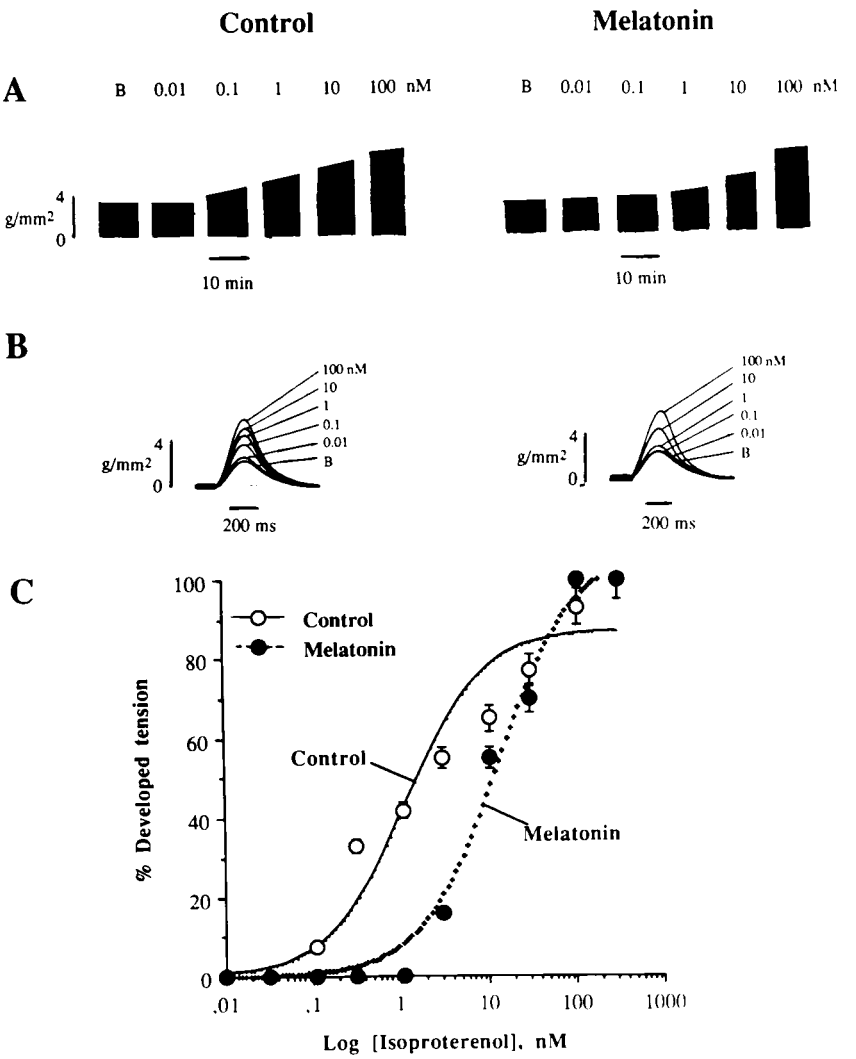

Fig. 2. Dose-response effects of isoproterenol (from 0.01 to 100 nM) in the absence (Control; left) and in the presence of melatonin (0.3 nM; right) on twitch tension of isolated rat papillary muscle (A). (B) Superimposed twitches obtained from the experiments illustrated in A. This figure is representative of data obtained in eight other experiments. (C) Dose-response curve to isoproterenol in the absence (Control) and in the presence of melatonin on percent developed tension. Results are the mean ± S.E. ($n = 8$).

Table 1
Kinetics of contraction and relaxation in rat isolated papillary muscle in the different procedures at maximum dose used

	Resting tension (g/mm^2)	Developed tension (g/mm^2)	Time to peak (ms)	Half contraction time (ms)	Half relaxation time (ms)
Control	2.1 ± 0.5	3.4 ± 0.4	113 ± 15	65 ± 7	105 ± 9
Melatonin (100 nM)	2.0 ± 0.4	3.3 ± 0.6	115 ± 18	68 ± 5	110 ± 8
Isoproterenol (100 nM)					
Control	2.4 ± 0.7	6.1 ± 0.7	87 ± 8	52 ± 5	81 ± 6
Melatonin	2.3 ± 0.8	5.9 ± 0.8	114 ± 6*	63 ± 5*	101 ± 7*
$[Ca^{2+}]$ (4 mM)					
Control	2.7 ± 0.6	7.3 ± 0.9	108 ± 11	62 ± 6	101 ± 5
Melatonin	2.6 ± 0.8	7.2 ± 1.0	111 ± 12	65 ± 4	106 ± 4
Isoproterenol (100 nM)					
Control	2.4 ± 0.4	6.2 ± 0.8	85 ± 6	54 ± 3	103 ± 6
Melatonin+*N*-acetyl-tryptamine	2.3 ± 0.5	6.0 ± 0.7	84 ± 9	51 ± 4	109 ± 4
Forskolin (10 μM)					
Control	2.5 ± 0.5	7.4 ± 1.0	93 ± 5	50 ± 5	84 ± 9
Melatonin	2.6 ± 0.5	4.1 ± 0.5*	112 ± 4*	66 ± 3*	108 ± 4*

Results are expressed as mean ± S.E.
*$p < 0.05$ vs. respective Control.

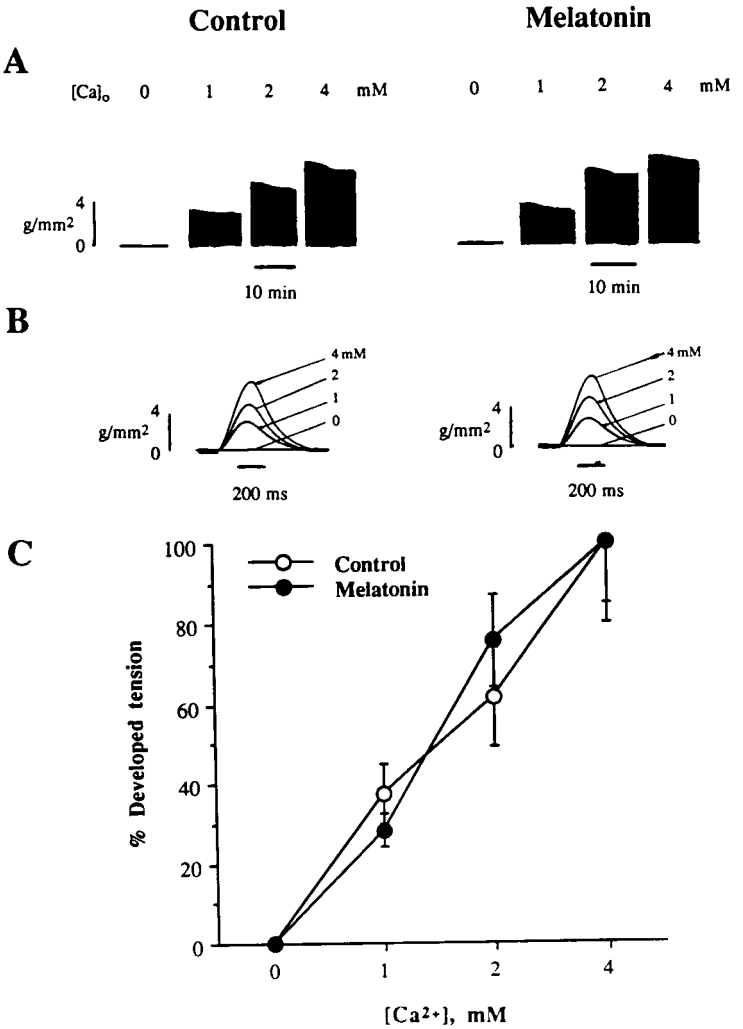

Fig. 3. Dose-response effects of $[Ca^{2+}]_0$ (from 0 to 4 mM) in the absence (Control; left) and in the presence of melatonin (0.3 nM; right) on twitch tension of papillary rat muscle (A). (B) Superimposed twitches obtained from the experiments illustrated in A. This figure is representative of data obtained in seven other experiments. (C) Dose-response curve to $[Ca^{2+}]_0$ in the absence (Control) and in the presence of melatonin on percent developed tension. Results are the mean ± S.E. ($n = 7$).

indicates that melatonin, at the highest dose used, did not show difference on kinetics of contraction and relaxation compared to control.

The β-adrenoreceptor agonist isoproterenol effects in the presence or in the absence of melatonin are shown in Fig. 2 ($n = 8$). Concentration response curve to isoproterenol on developed tension in controls shows a physiological trend beginning to increase at 0.1 nM. In contrast, in the presence of melatonin the curve was significantly shifted to the right beginning to increase at 3 nM. In addition, isoproterenol induced a reduction of time to peak, half contraction and relaxation time (Table 1); these modifications were significantly reduced in the presence of melatonin. Table 2 shows that isoproterenol concentration required to obtain 50% of developed tension (EC_{50}) was significantly higher in the presence of melatonin ($p < 0.001$).

In order to investigate whether melatonin effect on contractile response to isoproterenol was mediated by a direct action on calcium-dependent contractile mechanism, $[Ca^{2+}]$ concentration curves were performed. Fig. 3 depicts that increasing developed tension to $[Ca^{2+}]$ was similar between controls ansd melatonin-treated papillary muscles ($n = 7$). No difference was estimated in EC_{50} for calcium concentration curves both in the presence or absence of melatonin (p = N.S.; Table 2) indicating that melatonin action on contractile response was mediated by a calcium-independent mechanism.

Therefore, in order to investigate whether melatonin acts through a specific receptor interaction, we tested the effects of the specific melatonin receptor antagonist *N*-acetyltryptamine. The antagonist prevented melatonin from shifting the EC_{50} and kinetic contraction modifications induced by isoproterenol and (Fig. 4, Tables 1 and 2).

Table 2
EC_{50} for isoproterenol in controls, and in the presence of melatonin alone, or plus the specific receptor antagonist *N*-acetyltryptamine (10 μM), and for $[Ca^{2+}]$ in controls, and in the presence of melatonin

	Isoproterenol (nM)	$[Ca^{2+}]$ (mM)
Control	1.16 ± 0.12	1.63 ± 0.19
Melatonin	12.98 ± 1.68*	1.52 ± 0.16
Melatonin+*N*-acetyltryptamine	1.19 ± 0.14	

Results are the mean ± S.E. of different experiments (see Section 3).
*$p < 0.001$ vs. Control.

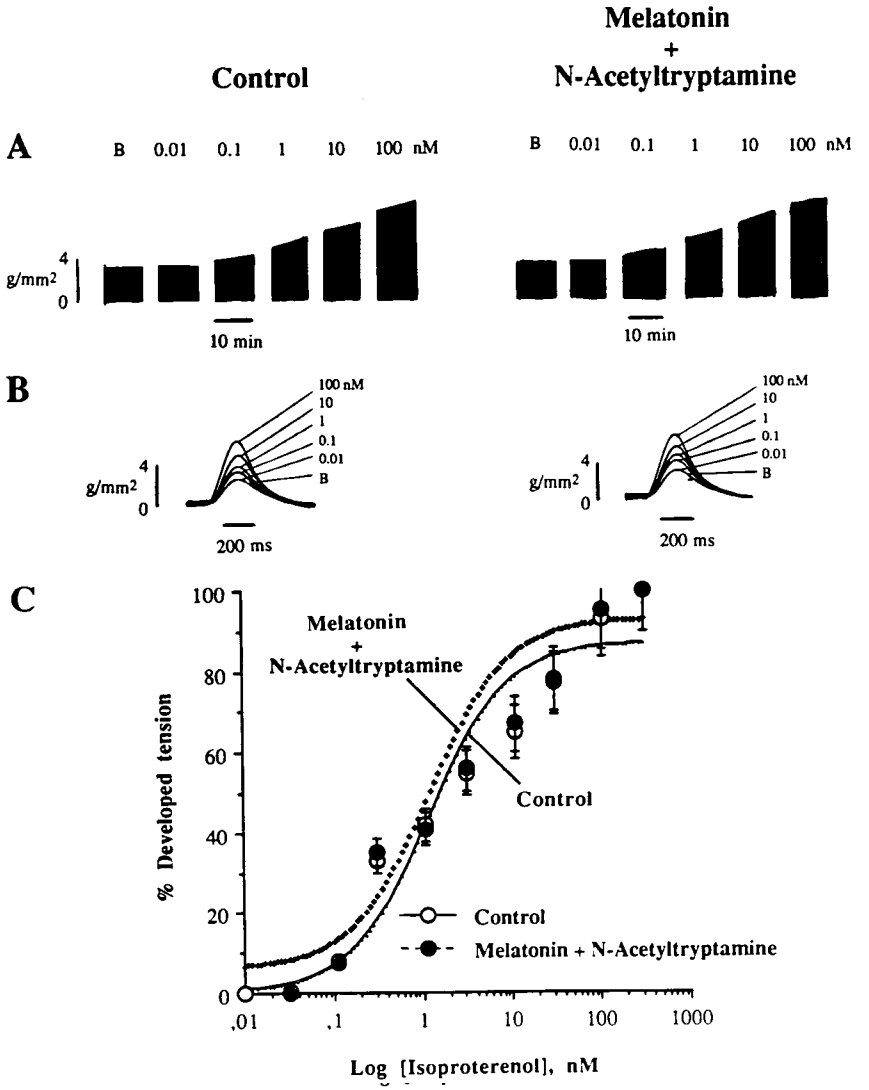

Fig. 4. Dose-response effects of isoproterenol (from 0.01 to 100 nM) in the absence (Control; left) and in the presence of melatonin (0.3 nM; right) plus its specific receptor antagonist *N*-acetyltryptamine (10 μM) on twitch tension of papillary rat muscle (A). (B) Superimposed twitches obtained from the experiments illustrated in A. This figure is representative of data obtained in eight other experiments. (C) Dose-response curve to isoproterenol in the absence (Control) and in the presence of melatonin plus its specific receptor antagonist *N*-acetyltryptamine on percent developed tension. Results are the mean ± S.E. ($n = 7$).

The mechanism of this receptor-interaction mechanism was also investigated by the stimulation of adenylate cyclase, and therefore cAMP accumulation, by forskolin. This set of experiments showed that forskolin increased the force of contraction in a similar fashion as isoproterenol (+118 ± 25%). This phenomenon was significantly reduced in the presence of melatonin (+26 ± 10%; $p < 0.01$) (Fig. 5, Table 1).

4. Discussion

Our results demonstrate that melatonin has no direct inotropic effect in isolated rat papillary muscle. More importantly is that melatonin counteracts isoproterenol but not [Ca^{2+}] effect. The anti-adrenergic action of melatonin in cardiac muscle occurs through an interaction to a specific cardiac receptor. The relative reduction of contractility increase, induced by forskolin-stimulated adenylate cyclase, shows that melatonin may act through a reduction of cAMP accumulation. The results of the present study confirm the anti-adrenergic action of melatonin found in other tissues [8–11].

Melatonin participates in several physiological functions, such as the control of seasonal reproduction as well as the immune system [6,7]. Experimental evidence suggests that melatonin acts by suppressing peripheral sympathetic activity and reducing noradrenaline turnover in the heart [20]. In addition, melatonin reduces norepinephrine and epinephrine content in adrenal gland [21]. Administration of melatonin in vivo determined a reduction of heart rate in baboon [22] and both arterial pressure and heart rate in rats [13] as the result of a hypothetical sympathetic inhibition. In fact, melatonin has been proposed as endogenous hypotensive factor probably by stimulating central inhibitory adrenergic pathways [23]. Our results firstly demonstrate that melatonin exerts a specific sympathetic antagonism in rat isolated papillary muscle which is an experimental model where central nervous system control and other hormonal influences are excluded.

Melatonin has two specific receptors (Mel_{1a} and Mel_{1b})

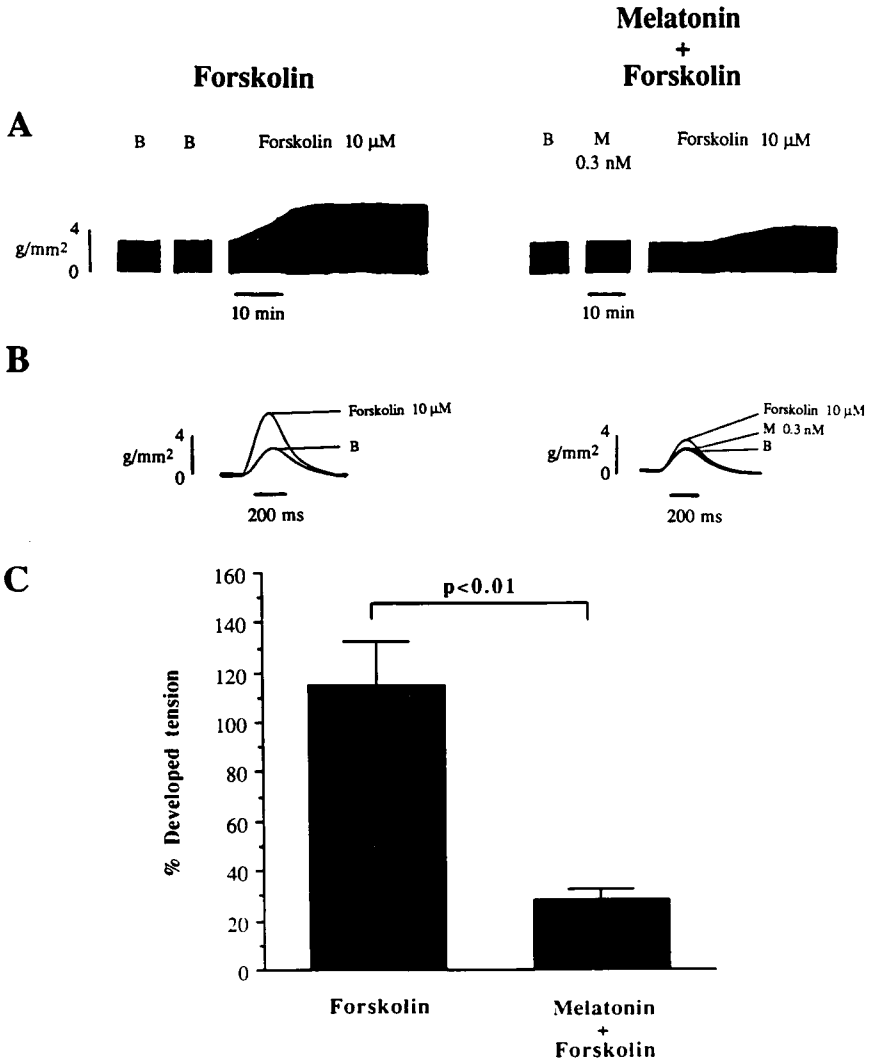

Fig. 5. Effects of 10 μM forskolin in the absence (forskolin; left) and in the presence of melatonin (0.3 nM; right) on twitch tension of papillary rat muscle (A). (B) Superimposed twitches obtained from the experiments illustrated in A. This figure is representative of data obtained in eight other experiments. (C) Effects of forskolin in the absence and in the presence of melatonin on percent developed tension. Results are the mean ± S.E. ($n = 8$).

linked to G-protein system in various tissues such as retina, brain, liver, spleen, and pituitary gland [8,9]. These receptors are coupled to inhibition of adenyl-cyclase, and, therefore, may negatively modulate the adrenergic system [8,9]. Furthermore, melatonin receptors are coupled to inhibitory G proteins to cyclic AMP accumulation [10] and to dopamine receptor-regulated adenylate cyclase [11]. The presence of a melatonin cardiac receptor is not well established. In a recent report, specific 2-[125] iodomelatonin binding sites were demonstrated in heart membrane preparations [12]. In order to examine the mechanism of action of melatonin at the level of receptor coupling on isoproterenol response, we used the specific receptor antagonist *N*-acetyltryptamine [24]. We found that melatonin effects on isoproterenol response was abolished in the presence of its receptor antagonist. This demonstrates that melatonin operates through a specific cardiac receptor demonstrating the presence of melatonin receptor in cardiac muscle similarly to other tissues [8,9]. A more probable explanation for the mechanism of receptor-interaction is the coupling of a melatonin receptor in papillary muscle to adenylate cyclase via an inhibitory G_i protein. In this scenario, melatonin, by activating Gi protein, would prevent the activation of adenylate cyclase by isoproterenol, and prevent a subsequent rise in cyclic AMP by isoproterenol, as previously showed [18,19]. This mechanism, in turn, would lower the efficacy of isoproterenol in inducing contraction in cardiac tissue. To test this hypothesis, we investigated whether the adenylate cyclase stimulator forskolin [25] increases the force of contraction in a similar fashion as isoproterenol, and successively, we tested the action of melatonin in this mechanism. We demonstrated that this hypothesis was correct. In fact, forskolin increased the force of contraction in a similar fashion as isoproterenol while melatonin reduced its action.

In conclusion, we found that melatonin possess anti-adrenergic effect in cardiac tissue. This was abolished in the presence of its receptor antagonist *N*-acetyltryptamine demonstrating that melatonin acts through a specific cardiac receptor. The reduction of contractility increase, induced by forskolin-stimulated adenylate cyclase, shows that melatonin

may exert its action through a reduction of cyclic AMP accumulation.

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